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Determination of protein *S*-acylation state by enhanced acyl-switch methods

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Running head: Improved acyl-switch assays using maleimide scavenging

Abstract:

S-acylation is increasingly being recognized as an important dynamic post-translational modification of cysteine residues in proteins. Various approaches have been described for assaying protein S-acylation with acyl-switch approaches being the most common and accessible. However, these approaches can be time consuming with low reproducibility as a result of multiple protein precipitation/resuspension clean-up steps. Here we present a faster, cleaner and more sensitive acyl-switch approach for detecting the S-acylation state of any protein, from any cell or tissue type, that can be detected by western blotting. In the case of acyl-RAC, the procedure is now performed without protein precipitation, greatly increasing speed and improving sample handling in the assay. This also allows for more samples to be processed simultaneously and opens the way for medium-throughput assays. Overall, maleimide scavenging improves the reliability of determination and quantification of protein S-acylation state by acyl-switch methods.

Key words (5-10): S-acylation, S-palmitoylation, S-acylated, palmitoylated, N-ethylmaleimide, acyl-switch, maleimide, 2,3-dimethyl 1,3-butadiene, biotin, Diels-Alder

1. Introduction:

S-acylation is an important post-translational modification occurring on cysteine residues in many proteins. S-acylation has a number of described roles, from controlling protein localization within the cell, anchoring soluble proteins to membranes, affecting protein stability and controlling the activation state of proteins. Current assays for assessing protein S-acylation commonly rely on two main approaches; orthogonal labelling with alkyne or azide functionalized fatty acids or hydroxylamine mediated acyl-exchange assays. Acyl-exchange assays rely on N-ethylmaleimide to

block free cysteine sulfhydryls and prevent non-specific detection [1]. This is followed by chemical cleavage of S-acyl groups to reveal free cysteine sulfhydryls that can be either labelled with (cleavable) biotin and selectively enriched using biotin-binding beads (typically streptavidin derivatives, acyl-biotin exchange; ABE) [2] or directly captured on sulfhydryl-reactive resin (acyl-resin assisted capture; acyl-RAC) [3]. Free sulfyhdryls can alternatively be labelled with PEG in PEG-shift/Acyl-PEG Exchange assays [4]. Acyl-exchange assays have historically involved numerous precipitation-based clean-up steps that lead to sample loss, protein aggregation and smearing of protein bands on gels. We recently described a faster, cleaner and more sensitive acyl-exchange assay specifically to address the issues we encountered with certain large, difficult or aggregation-prone proteins [5], but the method is equally applicable to all S-acylated proteins. Our novel approach is to use Diels-Alder 4+2 cycloaddition chemistry followed by phase partitioning to scavenge NEM from aqueous solution [6] rather than protein precipitation. As a result Acyl-RAC assays can now be performed without any precipitation steps being required, while ABE assays only require one precipitation step. In our hands this method greatly improves sensitivity and reproducibility while reducing hands-on time. Although not described here, the clean-up step to remove NEM should be equally applicable to PEG-shift/Acyl-PEG Exchange assays, or other assays in other fields requiring the removal of NEM from aqueous solution.

2. Materials:

Prepare all solutions using ultrapure water (at least 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Follow all local waste disposal regulations. The protocol assumes the ability to detect and quantify the protein of interest by SDS-PAGE and immunoblotting.

2.1 General solutions, reagents and equipment

1. 1 M N-ethylmaleimide (NEM) in ethanol (*see* **Note 1**)
2. Lysis buffer: 100 mM Tris-HCl pH7.2, 150 mM NaCl, 5 mM EDTA, 2.5 % SDS (*see* **Note 2**), 10 mM N-ethylmaleimide, protease inhibitors (*see* **Note 3 and 4**)
3. 1 M Hydroxylamine in water (pH to pH 7.2 with 10 mM NaOH) (*see* **Note 5**)
4. 1 M NaCl in water
5. 2,3-dimethyl, 1,3-butadiene
6. 2x reducing SDS loading buffer: 100 mM Tris-HCl pH 6.8, 4 % w/v SDS, 0.2 % bromophenol blue, 20 % v/v glycerol, 200 mM β-mercaptoethanol (store in 500 µl aliquots, add β-mercaptoethanol fresh)
7. BCA protein concentration determination kit (*see* **Note 6**)
8. Cooled benchtop microfuge (capable of ~ 16, 000 x *g*)
9. Cooled benchtop centrifuge (capable of > 4, 000 x *g* using 15 ml Falcon tubes)
10. Shaking heat block (*see* **Note 7**)
11. End-over-end 1.5 ml microfuge tube mixer (*see* **Note 7**)
12. 37 °C water bath

2.2 Acyl-biotin exchange (ABE)

1. Resuspension buffer: 100 mM Tris-HCl pH 7.2, 150 mM NaCl, 5 mM EDTA, 2.5 % SDS, 8 M urea (stored at -20°C in single use aliquots)
2. Chloroform
3. Methanol
4. Ultrapure water
5. 1mM biotin-HPDP dissolved in DMSO (prepare fresh immediately before use)
6. High-capacity Neutravidin-agarose beads

7. Phosphate-buffered saline (PBS) pH 7.2: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4. Prepare PBS by dissolving 8 g NaCl, 0.2 g KCl, 1.13 g Na_2HPO_4, 0.2 g KH_2PO_4, in 800 mL of H_2O. Adjust the pH to 7.2 ± 0.1 with 6 N HCl. Add H_2O to a final volume of 1 L.
8. ABE wash buffer: 1x PBS pH 7.2, 1% SDS
9. Roller mixer table

2.3 Acyl-Resin Assisted Capture (Acyl-RAC)

1. Acyl-RAC wash buffer: 100 mM Tris-HCl pH7.2, 150 mM NaCl, 5 mM EDTA, 2.5 % SDS (*see* **Note 8**)
2. Thiopropyl-Sepharose 6B beads

3. Methods

Initial sample processing (3.1) and sample analysis (3.3) is the same regardless of Acyl-RAC or ABE protocol. At step 3.2 follow either protocol 3.2a (ABE) or 3.2b (Acyl-RAC). Perform all steps at room temperature unless noted otherwise.

3.1 Sample lysis and free thiol blocking

1. If required prior to sample solubilisation, homogenise sufficient sample or tissue as appropriate for the species in question to provide ~1-2 mg total protein under conditions that minimize proteolytic degradation (*see* **Notes 2, 9 and 10**). For example, 15 10 day old *Arabidopsis thaliana* seedlings are ground to a fine powder under liquid nitrogen in a microfuge tube using a plastic micropestle and typically yields ~2mg total protein.
2. Add 500 µl lysis buffer to sample and mix gently at room temperature for 10 minutes until the tissue is thawed and/or fully solubilised in lysis buffer (*see* **Note 2**)
3. Centrifuge 1 minute at 16,000 x *g* to remove insoluble material.
4. Transfer supernatant to a fresh, labelled 1.5 ml microfuge tube.

5. Remove an aliquot to determine protein concentration by BCA assay.
6. Mix the remaining samples gently using end-over-end mixing.
7. Dilute samples to the same protein concentration, typically 0.25 - 2 mg/ml (*see* **Note 10**) in lysis buffer to a final volume of 1.1 ml.
8. Incubate samples at room temperature for 1h with gentle end-over-end mixing
9. Add 12 µl 2,3-dimethyl 1,3-butadiene (~100 mM final concentration) (*see* **Note 11**)
10. Mix samples for 1 h in a shaking hot block at 1500 rpm, 25 °C (*see* **Note 12**).
11. Briefly centrifuge to collect liquid.
12. Add 1/10th volume chloroform
13. Shake samples for 5 minutes in a shaking hot block at 1500 rpm, 25 °C (*see* **Note 13**).
14. Centrifuge the samples in a microfuge at 16, 000 x *g* for 1 minute to achieve phase separation (*see* **Note 14**)

3.2a Acyl-biotin exchange

15. Split the sample into two equal aliquots of 500 µl in fresh, labelled 15 ml conical centrifuge tubes
16. Add 500 µl 1M hydroxylamine pH 7.2 to one aliquot (Hyd +) and 500 µl 1 M NaCl to the other (Hyd -). Mix well without foaming (*see* **Note 15**).
17. Add 100 µl biotin-HPDP solution to both aliquots.
18. Mix the samples gently at room temperature for 1 h on a roller mixer table.
19. Chloroform/methanol precipitate the proteins [7]: add 1 vol chloroform to each aliquot and vortex. Add 4 vols methanol and vortex. Add 3 vols water and vortex.
20. Centrifuge at > 4, 000 x *g* for at 15 °C until phase separation is achieved. (*see* **Note 16**)
21. Remove and discard the clear upper phase without disturbing the protein pellet at the interface.

22. Add 4 vols methanol and gently mix. Take care not to lose any protein through dispersion up the side of the tube (*see* **Note 17 and 18**).
23. Centrifuge at 4, 000 x *g* for 30 minutes at 15 °C
24. Remove and discard supernatant and air dry the pellet (*see* **Note 19**).
25. Resuspend the pellets in 200 µl resuspension buffer (*see* **Note 20**).
26. Centrifuge tubes at 4, 000 x *g* to collect and clear the sample at the bottom of the 15 ml falcon tube.
27. Remove 10 µl from each hyd + and hyd - aliquot to a new tube for a loading control (LC + and LC - respectively). Add 10 µl 2x SDS loading buffer to each LC tube. Store samples at -20 °C until use.
28. Transfer 185 µl of each sample to a fresh labelled (EX + and EX -) 1.5ml microfuge tube and dilute with 1275 µl 1 x PBS. Avoid transferring any insoluble pellet.
29. Add 40 µl of a 50 % Neutravidin-agarose beads suspension to each EX+ and EX- and incubate at room temperature for 1 h with gentle mixing (*see* **Note 21**).
30. Collect the beads by centrifugation at ~1, 000 x *g*
31. Wash the beads x 3 with 1 ml ABE wash buffer and then x 2 with 1 ml 1 x PBS for 5 minutes each.

3.2b Acyl-RAC

1. Split the sample into two equal aliquots of 500 µl in fresh, labelled 1.5 ml microfuge tubes
2. Add 500 µl 1M hydroxylamine pH 7.2 to one aliquot (hyd +) and 500 µl 1 M NaCl to the other (hyd -). Mix well without foaming (*see* **Note 15**).
3. Remove 10 µl from each hyd + and hyd - aliquot to a new tube for a loading control (LC+ and LC- respectively). Add 10 µl 2x SDS loading buffer to each LC tube. Store samples at -20 °C until use.

4. Add 40 µl of a 50 % Thiopropyl-Sepharose beads suspension to each EX+ and EX- and incubate at room temperature for 1 h with gentle mixing (*see* **Note 22**).
5. Collect the beads by centrifugation at ~1, 000 x *g*
6. Wash the beads three times with 1 ml Acyl-RAC wash buffer.

3.3 Sample elution and analysis

1. Aspirate the beads to dryness after the final wash taking care not to remove any beads (*see* **Note 23**).
2. Elute the samples from the beads by adding 25 µl 2x reducing SDS loading buffer.
3. Heat samples at 37 °C for 30 minutes (*see* **Note 24**)
4. Analyze samples by SDS-PAGE and immunoblotting as appropriate for the protein of interest (*see* **Note 25** and **26**).
5. For a protein to be considered S-acylated, signal should be detected in the EX+ lane but not the EX- lane. Equal signal should be detected in both LC + and LC- lanes as shown in Fig. 1 (*see* **Note 27**).

4. Notes

1. N-ethylmaleimide solutions should be prepared fresh in dry ethanol. NEM is unstable in aqueous solutions and hydrolysis is accelerated by elevated pH.
2. If cells/tissue used produces viscous DNA contamination from lysis of nuclei by SDS, substitute 0.5% Triton X-100 or IGEPAL CA-630 for SDS in lysis buffer. Using ice cold 0.5% Triton-X100 lysis buffer, lyse the cells on ice for 1 minute with gentle mixing, centrifuge at 5000 x *g* 4 °C for 1 minute to pellet nuclei and add the supernatant to 1/3 volume of 10% SDS containing 10mM NEM.
3. S-acylation is very sensitive to the presence of reducing agents in buffers. Do not include reducing agents, particularly thiol-based ones, in lysis buffer unless absolutely essential. If essential use the absolute minimum or consider substituting for TCEP (Tris(2-

carboxyethyl)phosphine hydrochloride) as this phosphine based reducing agent is reported to be compatible with S-acylation at up to 5mM [8]. NEM will also react with TCEP and thiol-based reducing agents, eliminating them from solution and reducing the effective NEM concentration. If using reducing agents lysis buffer should not contain NEM, rather NEM should be added after reducing agents have had their desired effect. Reducing agent concentration should also be accounted for when adding NEM. Some protein quantification assays, such as BCA assays proposed for use here, are also sensitive to the use of reducing agents.

4. Use protease inhibitors appropriate to the species/system under study. In some cases the use of phosphatase inhibitors may also be appropriate.
5. Make hydroxylamine solutions immediately before use. Hydroxylamine solutions are unstable and degrade over time. As a result of degradation the pH of the solution elevates leading to undesirable side reactions.
6. Other protein concentration determination methods are available and suitable but must be compatible with the reagents used, in particular detergents.
7. Sample mixing requires both prolonged vortexing and gentle mixing. A range of lab-based equipment is suitable and specific types are not essential to the protocol.
8. This is the same buffer as lysis buffer but without NEM or protease inhibitors.
9. The variety of sample types to be analysed makes detailed instructions at this stage impossible. As a general rule use species-appropriate protocols designed for maximum disruption of cells/tissue for denaturing protein extraction.
10. Typically 250-1000 µg of total protein is required to perform an S-acylation assay depending on abundance of the protein of interest. We recommend starting with 10-20 times the mass of protein from which the protein of interest can be reliably and quantitatively detected by western blot.

11. 2,3-dimethyl 1,3-butadiene is volatile, flammable and has an unpleasant odor and should only be used in a fume hood. Store 2,3-dimethyl 1,3-butadiene at 4 °C to minimize volatilization.
12. Samples should be vigorously mixed (vortexed) to ensure dispersal of 2,3-dimethyl 1,3-butadiene throughout the aqueous phase.
13. Samples should form a visible emulsion but not foam. Adjust shaking speed accordingly.
14. Do not disturb the thin white layer of detergent formed at the chloroform/aqueous interphase.
15. We occasionally observe a white precipitate forming in some samples when using high protein concentrations, cool buffers or if lab temperatures are low. Briefly warming the sample to 37°C with gentle mixing will resolubilise the precipitate. Treat all samples identically even if they do not contain visible precipitate. No deleterious or unexpected effects or variations on downstream processing or results have been observed as a result of this precipitate.
16. We prefer to centrifuge for 20-30 minutes at 4000 x *g* using a swinging bucket rotor to ensure that the interphase remains undisturbed. Centrifugation using a fixed angle rotor allows for faster processing at higher speeds (protocol tested at 20,000 x *g* in a 45° fixed angle rotor) but care must be taken to avoid disturbing the interphase. Following centrifugation the upper phase should be completely clear, if it is still showing evidence of cloudiness repeat the centrifugation step.
17. Pipette addition of methanol followed by gentle swirling is usually sufficient to achieve mixing. Take care not to disperse sample up the side of the tube.
18. For dilute samples incubation at -20 °C for 1 hour to overnight can improve sample recovery. In our experience samples containing >100 µg/ml protein do not require this additional step for quantitative recovery.

19. Do not over-dry the pellet as this will hinder resolubilization. The pellet is typically dry of methanol and chloroform within 10 minutes. Some water will remain behind and the pellet should appear damp but this will not interfere with downstream steps.
20. To aid resuspension, samples can be alternately heated to 37 °C for 10 minutes in a water bath and mixed on a roller mixer table for 10 minutes. Careful vortexing can also help but should be done with care to avoid sample loss. Improper resuspension can result in either sample loss or “smearing” of samples on SDS-PAGE gels.
21. Prepare High-capacity Neutravidin beads by washing 3x with >10 bead volumes PBS and resuspending as a ~50% suspension in PBS for use.
22. Prepare Thiopropyl-Sepharose beads by hydrating in Acyl-RAC wash buffer for 15 minutes followed by 3 washes with >10 bead volumes PBS and resuspending as a ~50% suspension in Acyl-RAC wash buffer for use.
23. Aspirate buffer from the tubes until the buffer meniscus is just above the beads. Using a 10µl pipette tip mounted on a p20 pipette set to 20µl draw up buffer. Expel the buffer with some force to resuspend the beads and place the pipette tip immediately against the base of the tube. Gently aspirate the remaining buffer, taking care to maintain the seal at the bottom of the tube, and wipe off any adherent beads from the pipette tip on the side of the tube.
24. For large, heavily glycosylated or aggregation-prone proteins, heating samples to 95°C can result in smearing of samples on SDS-PAGE gels, particularly after the processing steps described here. Heating samples to 37 °C for 30 minutes or 65 °C for 15 minutes with occasional gentle mixing can help reduce smearing. We routinely use 37 °C for 30 minutes for all proteins without issue and have found elution to be more consistent without affecting protein mobility on SDS-PAGE gels.
25. We typically load all EX samples on one gel and all LC on another in order EX+, EX- or LC+,LC- for each sample. See figure 1 for example running order.

26. For fully quantitative analysis load the entirety of the sample, including beads, using a 200µl pipette tip. Loading the sample and beads will require a 1.5mm thick SDS-PAGE gel to accommodate the ~40 µl sample volume. Fill in empty wells with 2x SDS loading buffer to maintain consistent lane widths across the gel. Use appropriate quantification equipment and software designed for quantification of western blots rather than scanned film exposures.
27. If no signal is found in EX+ lanes this indicates the protein of interest is not S-acylated. If a signal appears in both EX+ and EX- lanes this indicates improper blocking of free sulfhydryls. No signal in LC lanes indicates either sample loss or the protein of interest is not expressed highly enough for detection by western blot. In either of these two latter cases, the experiment must be repeated for a conclusion to be drawn.

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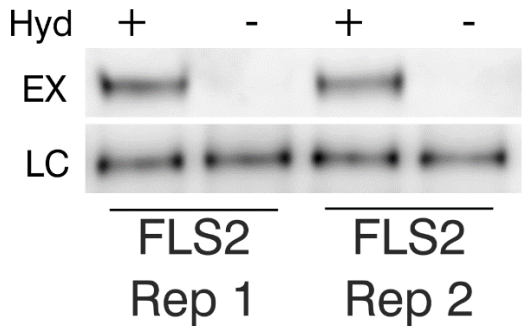

Fig. 1. S-acylation state of *Arabidopsis thaliana* FLS2 as determined by western blotting of samples processed by acyl-biotin exchange. The same initial sample was split in two and processed in parallel as technical replicates (Rep 1 and Rep 2). Hydroxylamine-treated samples: Hyd+, NaCl treated samples: Hyd-, EX: eluate from neutravidin beads, LC: loading control from before binding to neutravidin beads.